

ORIGINAL ARTICLE

Comparison of the Effectiveness of Autologous Fibrin Glue and Macroporous Biphasic Calcium Phosphate as Carriers in the Osteogenesis Process With or Without Mesenchymal Stem Cells

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Background: Facial bone reconstruction has been a challenge for oral and maxillofacial surgeons for a long time. Recently, some studies have reported the use of stem cells in facial reconstruction to achieve osteogenesis. However, to ensure that stem cells remain in the recipient site, a biocompatible carrier is needed to transfer the stem cells. Fibrin glue has been shown to promote hemostasis in wound management and accelerate soft tissue healing, but the role of fibrin glue in bone regeneration remains debatable. The purpose of this study was to compare the effectiveness of autologous fibrin glue and macroporous biphasic calcium phosphate (MBCP) as carriers in the osteogenesis process with/without mesenchymal stem cells.

Methods: Fifteen New Zealand white rabbits were used in this study. Mesenchymal stem cells were harvested from the iliac bone, and autologous fibrin glue was made from peripheral blood. Three cranial defects with a diameter of 6 mm were created over the cranial bone in each rabbit. The 15 animals were separated into 2 groups. The first group contained 12 rabbits. The grafted substances placed over the regions of defect were: (1) stem cells plus autologous fibrin glue; (2) stem cells plus MBCP; (3) defect alone as control. In the second group of 3 rabbits, the cranial defects were grafted with: (1) autologous fibrin glue alone; (2) MBCP alone; (3) defect alone as control. Rabbits were sacrificed at 1, 2 and 3 months post operation. Radiography and histology were used to detect bone formation.

Results: Stem cells plus autologous fibrin glue induced more bone formation 2 months post operation and more mature bone was found 3 months post operation compared with the other groups. MBCP with or without stem cells showed moderate tissue reaction, including giant cell, histiocyte and eosinophil cell accumulation.

Conclusion: Using stem cells plus autologous fibrin glue as the carrier may accelerate new bone regeneration. [*J Chin Med Assoc* 2008;71(2):66–73]

Key Words: autologous fibrin glue, biphasic calcium phosphate, osteogenesis, stem cells

Introduction

Repairing facial bone defects, either caused by congenital deficiency, trauma or tumor ablation, has been a challenge for oral and maxillofacial surgeons for a long time. Several techniques have proven successful. However, complications such as donor site morbidity

and chronic pain have been reported with the use of autograft material. The use of tissue-engineering triad concepts, including stem cells with osteogenic potential, scaffold and growth factor, to achieve osteogenesis in facial and cranial bone reconstruction is now under extensive study. However, to ensure that stem cells remain in the recipient site, a biocompatible carrier



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is needed to transfer the stem cells. Recently, some oral and maxillofacial surgeons have tried to implant bone marrow, platelet-rich plasma or stem cells to facial bone defects along with other substances, i.e. hydroxyapatite (HA), tricalcium phosphate (TCP), macroporous biphasic calcium phosphate (MBCP), or fibrin glue, to achieve bone regeneration.¹⁻⁵

MBCP is composed of 60% HA and 40% TCP. HA provides long-term stability and TCP releases ions that form acellular apatite crystals. The macroporosity of MBCP allows cells to penetrate material to a thickness of 2–3 mm.⁶⁻⁹ One animal study evaluated MBCP grafted with bone marrow in a high-dose-irradiated area. The result showed 6-fold new bone formation compared to MBCP alone.⁶

Fibrin glue application to decrease intraoperative bleeding and promote hemostasis has been used widely. Autologous fibrin glue contains 2 major components: fibrinogen and thrombin.¹⁰⁻¹² Fibrinogen is an abundant plasma protein, produced in the liver. The intact protein has a molecular weight of 340 kD. It is composed of 3 pairs of disulfide-bound polypeptide chains named alpha, beta and gamma. Proteolysis by thrombin results in fibrin monomers that are polymerized in a half-overlap fashion to form insoluble fibrin fibrils. The other major component in autologous glue is thrombin. Thrombin is an endoprotease that naturally functions as a blood clotting factor to convert fibrinogen to fibrin. Within seconds after fibrinogen and thrombin are mixed together, they form a gel-like component.¹⁰⁻¹²

Although the role of fibrin glue alone in interosseous and osteochondral healing remains debatable,^{13,14} it is now administrated as a scaffold in some tissue engineering triads *in vitro* and *in vivo*.

Stem cells may differentiate into osteogenic cells by some growth factors. Insulin-like growth factor and bone morphogenic proteins help regenerate bone.^{15,16} Other factors that enhance soft tissue healing are those rich in platelets. Platelets present huge reservoirs of growth factors for bone regeneration. Platelet-derived growth factor (PDGF) may initiate connective tissue healing.¹⁷ PDGF also increases mitogenesis, angiogenesis and macrophage activation.^{15,18-20}

Another growth factor is transforming growth factor- β , which increases the chemotaxis and mitogenesis of osteoblast precursor cells and also stimulates osteoblast deposition.^{21,22} However, these growth factors are only effective for a short period after transplantation *in vivo*.¹⁵

The purpose of the present study was to compare the effectiveness of autologous fibrin glue and MBCP as carriers in the osteogenesis process with/without rat mesenchymal stem cells (rMSCs).

Methods

Animal preparation

Fifteen New Zealand white rabbits, average weight 2.5–3.2 kg, were used in this study, which was approved by Taichung Veterans General Hospital's Animal Research Committee.

Mesenchymal stem cell preparation

Rabbit bone marrow was harvested from the iliac bone by using a bone needle with heparin-containing saline. The mononuclear cells were isolated from the whole marrow sample by centrifugation across a Ficoll-Hypaque gradient (Sigma, St Louis, MO, USA), and were resuspended in α -minimal essential medium containing 20% fetal bovine serum (Flow Laboratories, Rockville, MD, USA), L-glutamine and penicillin-streptomycin (Gibco BRL, Rockville, MD, USA). They were plated at an initial density of 2×10^4 cells/cm² for culture. The third generation of mesenchymal stem cells was used in the study. The concentration of cells was controlled at 1×10^7 cells/mL. Rabbit mesenchymal stem cells were then seeded onto 8-chambered slides (1×10^5 cells/well) (Corning, Invitrogen, Corning, NY, USA). The mesenchymal cells were identified by their surface phenotype: CD34(–), CD133(–) and CD105(+).

Autologous fibrin glue preparation

Ten mL of autologous blood drawn from each rabbit was combined with 1 mL of anticoagulant citrate dextrose phosphate to prevent coagulation. The blood was centrifuged at 1,500 rpm for 10 minutes at ambient temperature to separate the plasma containing the platelets from the red cells and buffy coat. Using a modified version of the autologous fibrin glue preparation method in Thorn et al's study,²³ we extracted the fibrinogen from the plasma using cold ethanol precipitation. Thrombin was produced from diluted plasma by precipitation of euglobulins at low pH. One-tenth of the platelet-rich plasma was separated for thrombin preparation. The euglobulin fraction was precipitated by dilution with citric acid. After thorough mixing of the solution, the tube was centrifuged at 3,000g for 5 minutes at 4°C. The supernatant was removed and the precipitate was dissolved in CaCl₂ (0.1 M). The pH was adjusted to neutral values by NaHCO₃, and the activation of prothrombin to thrombin was started. During the next 3–10 minutes, a fibrin clot was formed. The clot was gently squeezed during the gel formation process. After 20–30 minutes, clot formation was terminated and the liquid thrombin solution was removed by syringe. To avoid contamination,

these procedures were performed in a laminar air flow bench.

Animal surgical procedure

The rabbits underwent general anesthesia by intramuscular injection of atropine 0.1 mg/kg, ketamine 50 mg/kg and Romfom 5 mg/mL with standard monitoring. Each rabbit's fur was shaved over the operation site and draped in a sterile fashion. Mesenchymal stem cells and autologous fibrin glue were harvested from the iliac bone and peripheral blood. According to Aghaloo et al's study design,¹ 3 cranial defects with a diameter of 6 mm each were created over the cranial bone in each rabbit. The 15 animals were separated into 2 groups. The first group contained 12 rabbits. The grafted substances placed over the regions of

defect were: (1) stem cells plus fibrin glue; (2) stem cells plus MBCP (Biomatante, Vigneux de Bretagne, France); (3) defect alone. In the second group of 3 rabbits, the cranial defects were grafted with: (1) autologous fibrin glue alone; (2) MBCP alone; (3) defect alone (Figure 1). After implantation, the dura was first closed by using 4-0 vicryl to prevent graft material displacement, and 4-0 nylon was then used to suture the skin layer. The rabbits recovered without complications after anesthesia subsided.

Results

Clinical evaluation

All rabbits remained healthy before being sacrificed with intravenous pentobarbital 100 mg/kg at 1, 2 and 3 months post operation. Fibrous tissue was observed to have extended to the defect on clinical observation (Figure 2). The defect margin became difficult to identify 3 months after operation. MBCP particles remained in the cranial defect 3 months postoperatively.

Radiologic examination

Radiographs were taken of each rabbit's cranium before histologic sections were examined. The defects with graft implantation were increasingly radio-opaque at 1, 2 and 3 months after surgery. More radio-opacity presented in the MBCP group due to the MBCP particles that remained. The margin of the defect was difficult to identify 3 months postoperatively (Figure 3).

Histologic examination

Specimens were treated with hydrochloric acid decalcifying solution and then dehydrated with graded



Figure 1. Rabbit cranial defects. The grafted substances placed over the defects were: macroporous biphasic calcium phosphate particles (upper left); fibrin glue (upper right); defect alone (bottom).

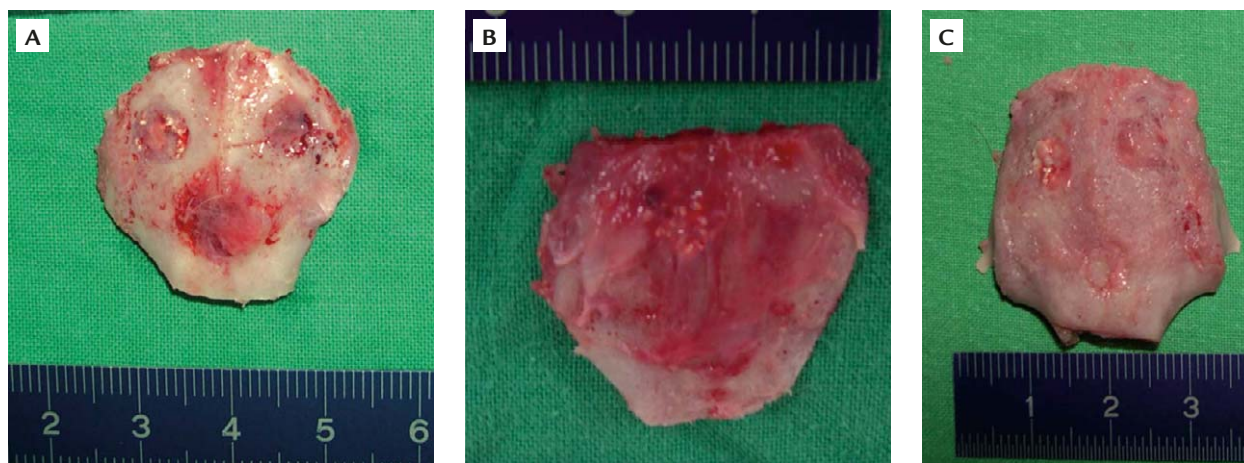


Figure 2. Cranial defects after sacrifice of the rabbits at postoperative: (A) 1 month; (B) 2 months; and (C) 3 months. Macroporous biphasic calcium phosphate particles are still present at 3 months post operation.

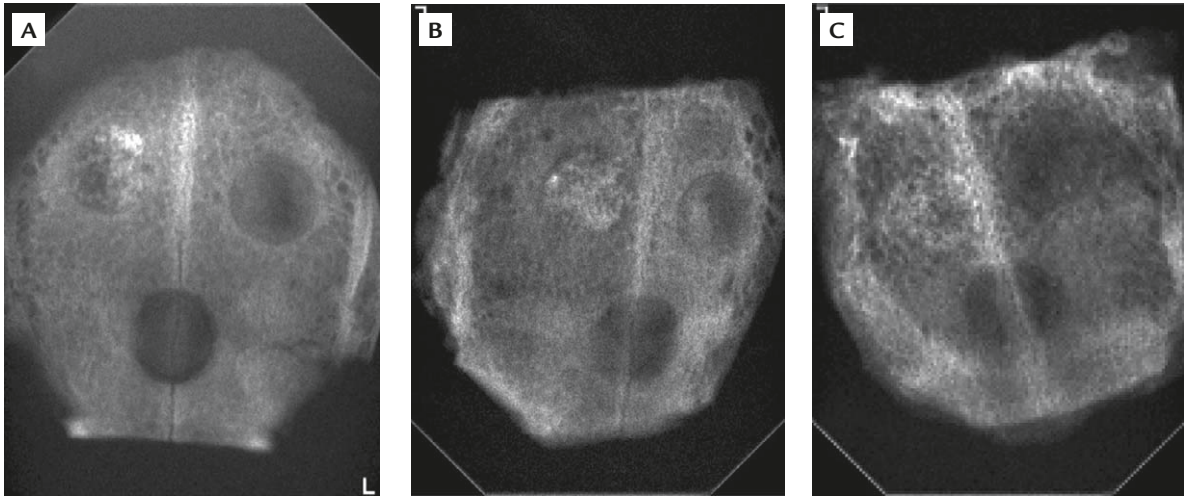


Figure 3. Radiographs of the rabbit craniums at postoperative: (A) 1 month; (B) 2 months; and (C) 3 months. Macroporous biphasic calcium phosphate (MBCP) particles are still present at 3 months post operation. There is increased radio-opacity in the MBCP+rat mesenchymal stem cells (rMSCs) (upper left defect) and fibrin glue+rMSCs (upper right defect), compared to the defect alone (bottom).

alcohols and embedded in paraffin. They were subsequently sectioned at 6 μm with a steel knife. Histologic specimens were prepared in the usual fashion with hematoxylin and eosin staining. Bone remodeling and regeneration were present around the peripheral cranial defect. In the fibrin glue plus rMSC group, more new bone formation was present compared to the control group and the fibrin glue alone group. Osteoblasts rimmed the peripheral defect and new bone formation at the central portion of the defect was noted in the fibrin glue plus rMSC group 2 months postoperatively. In addition, at 2 months post operation, residual fibrin glue was still present and mature lamellar bone formation was noted (Figures 4 and 5). The fibrin glue plus rMSC group had more bone formation compared to the other groups. Furthermore, in the MBCP plus rMSC group, there was inflammatory cell accumulation, including histiocytes, eosinophils and lymphocytes. The MBCP plus rMSC group showed more tissue reaction than the implantations of the MBCP-alone group. Maximum inflammation was noted at 2 months post operation. The inflammatory condition seemed to begin to subside 3 months postoperatively.

Discussion

The tissue-engineering triad concept has been employed in many animal studies and clinical practice to reconstruct maxillofacial defects. Different facial regions need different scaffold modalities.^{24–26} In von Arx et al's study, TCP demonstrated 70% new bone formation at 24 weeks, compared to 54% in the autografted site.²⁷ Alloplast material can be used to reconstruct the defect

and achieve new bone formation. However, many surgeons are still concerned about the biocompatibility of alloplast material. Several studies have investigated the inflammatory response to biphasic calcium phosphate, which is composed of HA and TCP.²⁸ Fibrous tissue encapsulation of biphasic calcium phosphate microparticles was observed in Fellah et al's study.²⁹ They found that micro biphasic calcium phosphate (pore size <20 μm) caused more inflammation and giant cell accumulation than the intermediate and macro pore sizes (40–80 μm and 80–200 μm , respectively). However, we found that MBCP plus rMSC could cause foreign body reaction, with accumulation of lymphocytes, plasma cells and histiocytes. MBCP still remained 3 months postoperatively.

Regarding cytotoxicity, some studies showed that due to the release of microparticles, cell damage occurred around the implanted biphasic bioceramic.^{30,31} In our study, stem cells did not present at 1 month after implantation with MBCP. Although MBCP may release microparticles that cause inflammation and impair cell vitality, our study showed that inflammation subsided after 3 months post operation. In another animal study, De Kok et al³² mixed mesenchymal stem cells with HA/TCP, and then placed the mixture in a beagle dog's extraction socket. Mild inflammation was present 7 days post operation, but had diminished by postoperative day 21. In our study, the rMSC plus MBCP group had delayed inflammation 2 months postoperatively. Viable cell adhesion on the biphasic calcium phosphate granules was low in some studies.^{29,31,32}

Fibrin glue contains approximately 10 times the concentration of fibrinogen, F-VIII, vWF, F-XIII and

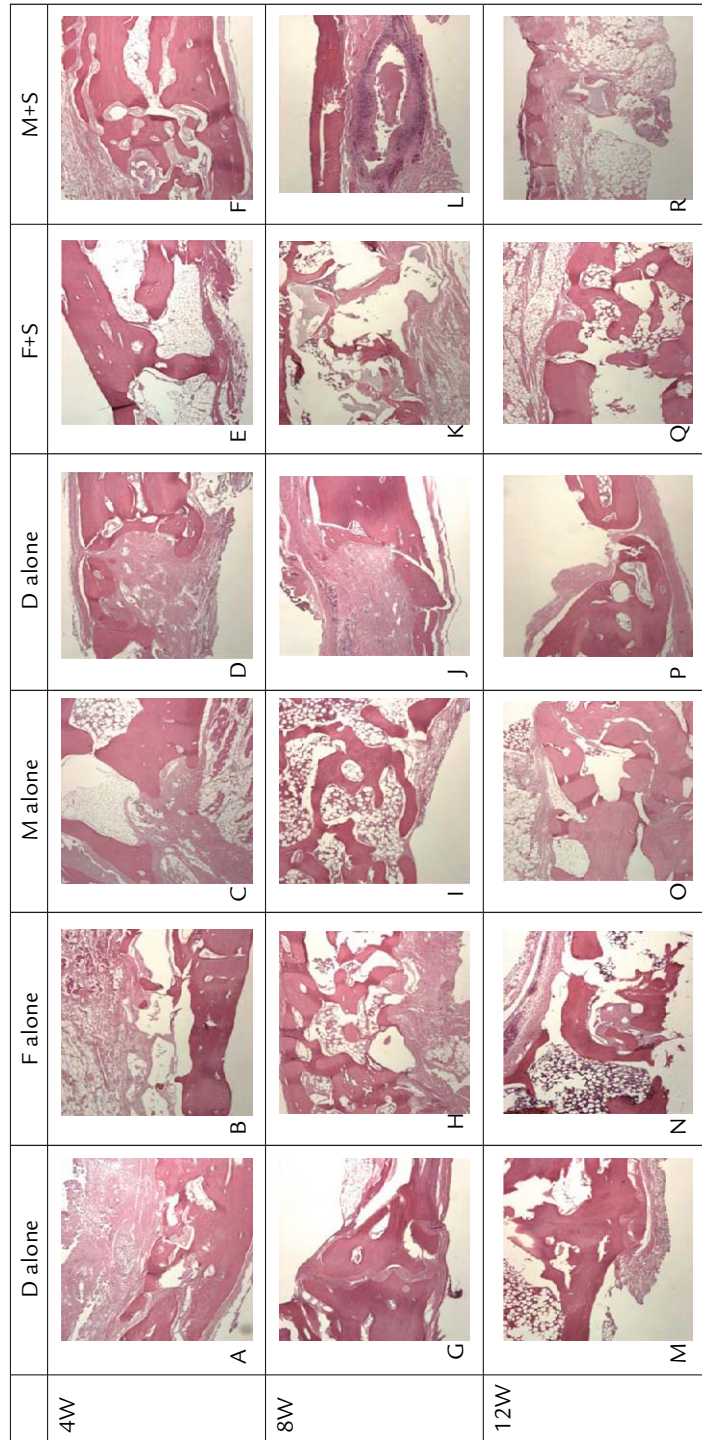


Figure 4. Histologic examination with hematoxylin and eosin staining; original magnification 40 \times . c = control; W = week; D = defect; F = fibrin glue; M = macroporous biphasic calcium phosphate; S = mesenchymal stem cells. (A) c4WD: fibrosis formation in bony defect. (B) c4WF: new bone formation from old bone, fibrin glue remains. (C) c4WM: new bone formation, interacting with MBCP; macrophage, tissue reaction present. (D) 4WD: fibrous tissue formation in defect. (E) 4WF+S: more new bone formation compared to fibrin glue alone; fibrin glue remains. (F) 4WM+S: new bone formation, interacting with MBCP; MBCP particles remain. (G) c8WD: new bone formation present from the periphery. (H) c8WF: new bone formation at the center of the defect, interacting with fibrin glue. (I) c8WM: new bone formation. (J) 8WD: new bone formation from the peripheral defect. (K) 8WF+S: new bone formation, interacting with fibrin glue; osteoblasts present at the periphery of new bone. (L) 8WM+S: histiocytes, eosinophils, lymphocytes and plasma cells encroach MBCP particles. (M) c12WD: new bone formation at the periphery. (N) c12WF: mature bone formation at the central defect, no fibrin glue remains. (O) c12WM: mature bone formation at the central defect. (P) 12WD: mature bone formation at the periphery. (Q) 12WF+S: mature new bone formation. (R) 12WM+S: new bone formation surrounded by lymphocytes and histiocytes.

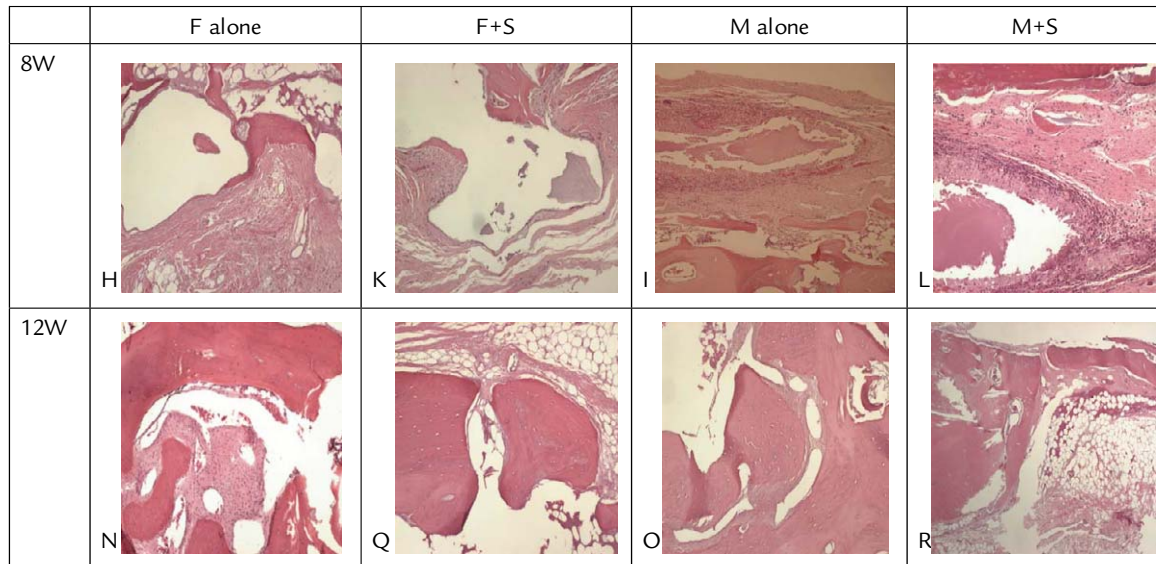


Figure 5. Histologic examination with hematoxylin and eosin staining; original magnification 100 \times . c = control; W = week; D = defect; F = fibrin glue; M = macroporous biphasic calcium phosphate; S = mesenchymal stem cells. (H) c8WF: new bone formation at the center of the defect, interacting with fibrin glue. (N) c12WF: Mature bone formation at the central defect; no fibrin glue remains. (K) 8WF+S: new bone formation, interacting with fibrin glue; osteoblast present at periphery of new bone. (Q) 12WF+S: mature new bone formation at the central defect. (I) c8WM: new bone formation. (O) c12WM: mature bone formation, connecting to peripheral bone. (L) 8WM+S: histiocytes, eosinophils, lymphocytes and plasma cells encroach MBCP particles. (R) 12WM+S: new bone formation, surrounded by lymphocytes and histiocytes.

fibronectin of fresh frozen plasma.^{9,33} One *in vitro* study showed that fibrin glue may increase cell-seeding efficiency to collagen scaffolds and cells may appear fibroblastic in shape. However, when fibrin glue alone was used as a scaffold, the cells were spherical.³⁴ An osteoblast rim around the new bone was also found. Whether fibrin glue alone can be a strong scaffold depends on its implantation location. In the rabbit cranial defects in our study, it was surrounded by hard tissue. Hard tissue may also enhance the scaffold potential of fibrin glue. Fibrin glue has no inhibitory effect on cell morphology, growth, proliferation or differentiation. It has good biocompatibility and can be used as scaffold material for bone marrow mesenchymal stem cells in bone tissue engineering.³⁵ The half life of the fibrin clot was around 4.6 days, and it had completely disappeared from the body within 30 days in some studies.^{36,37} One study claimed that commercially produced fibrin glue could degrade within 1–2 weeks.³⁷ However, in our study, residual fibrin glue still remained 2 months after implantation and no granulation tissue reaction was found (Figure 4). This may be because the fibrin glue had been placed in a defect surrounded by hard tissue, delaying its degradation.

Cloning experiments have shown that mesenchymal stem cells fail to proliferate beyond 20 population doublings.³⁸ When an adequate cell number has been

achieved, re-implantation to the defect should be performed. The stem cells were cultured *in vitro* before operation. The concentration of cells was controlled at 1×10^7 cells/mL. No rMSCs remained in the cranial defect 1 month postoperatively, and osteoblasts rimming the new bone in the central part of the defect were found 2 months postoperatively (Figure 5). Using stem cells in various ways to mobilize the damaged organ seems to be a promising modality.^{39–42} Some clinical studies used autologous peripheral blood stem cell transplantation, combined with chemotherapy/radiotherapy, to treat chemosensitive malignant disease (i.e. acute myeloid leukemia) and got good responses.^{43,44}

Osteoblasts can be enticed to move across a greater distance by creating a scaffold system. The movement of osteoblasts along the fibrin network results in the formation of woven bone much earlier than usual. Using fibrin glue as a scaffold plus rMSCs resulted in earlier and more mature new bone formation compared to using fibrin glue alone (Figure 5).

Researchers have tried using mixed scaffolds of fibrin glue with calcium phosphate ceramic granules or MBCP. In some studies, new bone formation was found on the periphery of the bony defect, and these scaffolds provided more stable structure.^{7,39} Although new bone formation was also found when using MBCP or MBCP plus rMSCs, inflammatory cell accumulation around the new bone also occurred. This can

compromise the quality of the new bone when further fixture implantation is considered. In Bluteau et al's study,⁹ osteoblasts covered the MBCP, which was coated with thrombin or fibrinogen. However, very few cells were observed on the surface of MBCP covered with Tissucol[®] on scanning electron microscopy study. The surface of MBCP needs to be coated with fibrinogen or thrombin to increase osteoblast adaptation. In clinical application, using 2 or more scaffolds increases the cost and difficulty of the operation. For a non-critical sized defect, using fibrin glue with stem cells can simplify the early osteogenic procedure.

The sample size in our study was small: only 15 rabbits were used. More inflammation was present in the MBCP alone and rMSC plus MBCP groups. Using fibrin glue with mesenchymal stem cells resulted in earlier and more mature new bone formation. In craniofacial bone reconstruction, autologous fibrin glue combined with osteogenic mesenchymal stem cells may have a promising role in the future.

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